Cycling mouse oocytes through meiosis

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Cell Cycle News & Views

Will helicases contribute to the fight against malaria?

Comment on: Tuteja R. Cell cycle 2010; 9:104-20.

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Helicases are enzymes involved in all aspects of nucleic acid metabolism.[1] Because of their essential function, helicases are ubiquitous and evolutionarily conserved proteins. Helicase s are characterized by the presence of conserved motifs in the form of short amino acid sequences. Based on variations of the number of motifs, their amino acid sequence and spacing, helicases have been grouped into superfamilies (SF), including three large (SF1–SF3) and two small (SF4 and SF5) ones.[2] The crystal structures of several representative helicases revealed common features. Monomeric helicases (SF1/SF2) have a core that consists of two domains with a linker region. Hexameric helicases (SF3-SF5) form a core that includes six individual domains arranged in a ring. The domains are termed RecA-like because of their similarity to the ATP binding core of RecA recombination protein. The conserved helicase motifs include those involved in NTP binding and hydrolysis, which are similar to the Walker A and B boxes of ATPase. The other conserved motifs are involved in coupling of the NTP hydrolytic state to protein conformational changes and in nucleic acid binding.[3]

Helicases utilize the energy derived from nucleoside triphosphate hydrolysis to translocate along nucleic acid strands, unwind/separate the helical structure of double-stranded nucleic acid and, in some cases, disrupt protein-nucleic acid interactions.[4] Depending on their nucleic acid targets, helicases are generally classified as DNA or RNA helicases. DNA helicases are involved in replication, repair and recombination. RNA helicases are involved in all aspects of RNA metabolism. The majority of RNA helicases belongs to DExD/H-box proteins which derive this name from the single letter code of the four amino acids of motif II.[5] They are classified into several subgroups including DEAD-box (DDX) and DEAH-box (DHX) families which are distinguished by consistent sequence differences that extend beyond motif II.[6]

In addition to the basic study of their biochemical and biophysical properties, interest in helicases includes their potential use as targets of novel therapies. An example is the research conducted to identify inhibitors of viral RNA helicases.[7] Although malaria is preventable and curable, it continues to be a major cause of morbidity and mortality. Plasmodium falciparum causes the most virulent form of malaria. The fight against malaria is complicated by the development of parasite resistance to currently used treatment modalities.8 Thus, there is a continuous search for novel targets of anti-malarial therapy. The question of whether helicases can contribute to our fight against malaria is one step closer to being answered. In the January 1, 2010 issue of *Cell Cycle*, Renu Tuteja from the International Centre for Genetic Engineering and Biotechnology in India reports a bioinformatics approach to identify members of several families of helicases in *Plasmodium falciparum*.[9] This study reveals the presence of members of the DEAD-box family of helicases that have no obvious human homologues. DEAD-box family is the largest family of RNA helicases which includes members involved in all aspects of RNA metabolism.[10] Previous work from the same laboratory showed that targeting a DEAD-box RNA helicases can impair parasite growth.[11] Identification of novel agents that can target Plasmodium falciparum-specific helicases with the aim of disrupting their life cycle with little side effects on the human host would be a novel contribution to our fight against malaria.

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The NF-Y angle to the *CCAAT*'s tale

Dolfini D, et al. Cell Cycle 2009; 8:4127-37.

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Regulation of gene-specific transcription in eukaryotes is controlled by combinatorial arrangements and concerted functions of various short DNA sequence elements located around the transcription start site (TSS) of genes (promoters) as well as in distal regulatory regions (e.g., enhancers) that are recognized by diverse sequence-specific DNA-binding proteins. The availability of completely sequenced genomes has promoted efforts to decipher by molecular and bioinformatics approaches the genome-wide location and combinatorial assortment of some of these well-characterized transcription regulatory sequences and cognate regulatory factors. In a previous issue of *Cell Cycle,* Dolfini et al. provide a new view of the type of promoters/genes controlled by the prototypical CCAAT box sequence and its associated ubiquitous NF-Y regulator. These and similar studies are essential to our understanding of how specific genes are regulated but also will facilitate in silico identification of classes of genes and other non-coding transcripts of unknown function that might be regulated via common pathways; this is particularly important given emerging evidence of widespread transcription throughout the genome, beyond the known/annotated (RefSeq) genes. [1-3]

The first conserved DNA elements shown to control eukaryotic gene transcription were identified in the promoter regions of a few cellular and viral protein-coding genes transcribed by mammalian RNA polymerase II (Pol II). These prototypical control elements include the notorious TATA and CCAAT boxes. The TATA box is a "core promoter element" located at -30 bp relative to the TSS (+1) that functions by recruiting the general/basal transcription initiation factor TFIID and thereby nucleates the assembly of the rest of the basal transcription machinery at the TSS; the CCAAT box is a "promoter-proximal" regulatory sequence that is found in either orientation upstream of the TATA box (in the -60/-200 bp region) and generally activates transcription.[4,5] The factors that specifically recognize the CCAAT box were initially thought to be multiple and included C/EBP, CTF/NF-I, NF-Y and others. Eventually the detailed characterization of the specific nucleotide sequence requirements for DNA binding by these different transcription regulators indicated that NF-Y is the major CCAAT-binding factor, which functionally recognizes not only the CCAAT pentanucleotide but also flanking nucleotides that perfectly match the statistical CCAAT consensus sequence.[5,6] NF-Y (also called CBF) is a heterotrimeric complex composed of NF-YA, and the histone fold domain NFY-B and NF-YC subunits, which are all required for DNA binding; notably, the nuclear localization of NF-YC is cell cycle-regulated and dependent on dimer formation with NF-YB.[7] While NF-Y is a "glutamine-rich" transcription activator that cooperates with neighboring promoter-bound factors and helps recruit the Pol II machinery, it is also associated with gene repression and negative histone methyl-marks.[8]

Early statistical and functional analyses suggested that the TATA and CCAAT boxes are present and important in most protein-coding gene promoters.[5] However, as many more promoters and 5' ends of mRNAs were accurately characterized and genomes sequenced, it became apparent that only a minority of eukaryotic promoters have a TATA box - only ~10-24% in humans.[9,10] Now, Dolfini et al., in a previous issue of *Cell Cycle*, uncover a similar under-representation of the prototypical CCAAT box in human promoters by using a refined Position-Specific Frequency Matrix (PSFM) derived from experimentally validated NF-Y/CCAAT sequences. They find that only ~12% of all RefSeq protein-coding genes in the human genome contain "high confidence" NF-Y/CCAAT boxes in their promoter region, significantly less than previous estimations using limited promoter datasets and earlier PSFMs.[5,11] Importantly, the low frequency of NF-Y/CCAAT boxes in promoters, is in agreement with recent "ChIP-on-chip" (chromatin immunoprecipitation and hybridization to DNA microarrays) experiments, which indicated binding of NF-YA and NF-YB to, respectively, ~10% of 16,695 promoters[12] and ~22% of 907 promoters[8] of human RefSeq genes.

Dolfini et al. further define the CCAAT box-containing promoters/genes and verify that CCAAT boxes typically occur in several copies, in either orientation, and preferentially in the -80 bp promoter region of genes that are often involved in cell cycle and chromatin/transcription, consistent with previous observations. More unexpectedly, however, CCAAT box-containing promoters generally lack a TATA box, have heterogeneous TSSs, and tend to be located within CpG islands. Thus, the traditional view of Pol II promoters having both TATA and CCAAT boxes, based on the original characterization of selected promoters, is very rare. These results also suggest a possible function of NF-Y/CCAAT boxes in facilitating the recruitment of the Pol II machinery to TATA-less promoters, perhaps in a manner similar to GC boxes and cognate glutamine-rich activator SP1. Notably, Dolfini et al. uncover that CCAAT box-containing promoters are enriched in sites for specific regulators such as SP1, E2F, CREB and USF/E-boxes, which are known to cooperate with NF-Y, and sites for potentially new interacting/cooperating regulators such as PAX, NFκB and ELK. Interestingly, the authors further document that multiple NFY/CCAAT boxes are typically separated by one, or several, integral DNA helical turns, and observe "preferred" spacing between NF-Y/CCAAT boxes and some of its cooperating regulators (SP1 and CREB), suggesting their global interactions.

In conclusion, the studies by Dolfini et al. provide a new global view of the organization of human RefSeq genes/promoters regulated by the prototypical NF-Y/CCAAT box and should help comparative studies of the structural and functional organization of all genomic loci bound by NF-Y. Indeed, it is intriguing that the vast majority (~80–90%) of NF-Y binding sites analyzed by ChIP-on-chip on chromosomes 20, 21 and 22 have been shown to reside outside of RefSeq promoters,[8] suggesting that this might also be the case genome-wide. Do these most frequent distal NF-Y/CCAAT sites also correspond to locations of promoters for novel mRNAs or non-coding RNAs,[13] or do they represent regulatory enhancer/silencer or chromatin boundary regions? Alternatively, could these sites reflect other non-transcription functions of NF-Y/CCAAT? Clearly, the future promises more twists and turns to the NF-Y/CCAAT's tale.

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Cycling mouse oocytes through meiosis

Comment on: Miles DC, et al. Cell Cycle 2010; 9:408-18.

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In eukaryotic species, cells divide by mitosis to clonally expand cell populations or by meiosis to generate genetically diverse gametes for sexual reproduction. However, despite its fundamental role in developmental biology, genetics and evolution, many aspects of the meiotic cell cycle are considerably less well understood than their mitotic counterparts. In mammalian species, the ability to divide by meiosis is restricted to germ cells, although the timing and regulation of meiosis differs between female and male germ cells. In females, proliferating germ cells initiate

the meiotic cell cycle en masse in the fetal ovary, pass through most of meiotic prophase, then arrest in the diplotene stage of meiotic prophase a few days after birth. In contrast, male germ cells become quiescent during late fetal development, and a continuous stream of proliferating male germ cells initiate and proceed through meiosis throughout adult life.[1] Although the differences in cell cycle behavior between male and female fetal germ cells have been known for some time, and are often used to distinguish whether fetal germ cells have embarked on male or female developmental programmes,[2] little is known about how the cell cycle is regulated to drive this sexually dimorphic behavior.

The study by Miles et al.[3] in the previous issue of *Cell Cycle* uses transgenic mice that express GFP in the developing germ cells to analyze the expression of cell cycle regulators in purified female germ cells during meiotic prophase. Miles et al. focus mainly on proteins such as cyclin B, and the DNA damage-associated kinases ATM and ATR that are known to be able to regulate progression through the G_2-M transition in mitosis.[3] The abundance of the active form of ATM, but not active ATR, in developing germ cells appears to be influenced by the sex-specific differences in cell cycle progression in fetal gonads.[3] The high levels of active ATM and its downstream target Chk2 in oocytes during the early stages of meiotic prophase in oocytes presumably reflects the generation of meiotic DNA double-strand breaks during leptotene that stimulate meiotic recombination and chromosome synapsis during zygotene and pachytene. The ATM pathway may help to monitor meiotic chromosome synapsis in oocytes by preventing cell cycle progression until chromosome synapsis repairs all the meiotic DNA double-strand breaks in a manner analogous to ATM-mediated checkpoints preventing the mitotic G_2-M transition in the presence of DNA double-strand breaks.[4]

Perhaps one of the most salient findings of the study by Miles et al. is that expression of the three murine paralogs of cyclin B are differentially regulated as oocytes pass through the first meiotic prophase to diplotene.[3] In particular, expression of cyclin B3, which has been previously characterised as a meiosis-associated cyclin B paralog, is upregulated during the leptotene and zygotene stages of meiosis.[3,5] In contrast, cyclin B1 is downregulated during early meiotic prophase while cyclin B2 expression remains relatively unchanged.[3] Interestingly, human cyclin B3 is able to form a complex with Cdk2, although these complexes do not have strong kinase activity in vitro.[5] It is not yet clear if the low kinase activity of cyclin B3-Cdk2 complexes reflects the absence of correct binding partners, modifications, or meiosis-specific substrates in the in vitro assay, or whether the low kinase activity of cyclin B3-Cdk2 complexes might be important for the prolonged duration of prophase in meiotic cells. Regardless, it will be of interest to determine whether cyclin B3 mediates the distinct localization of Cdk2 to meiotic chromosomes and/or the meiosis-specific requirement for Cdk2 in progression through meiotic prophase.[6,7] The expression of different cyclin B paralogs during mitosis and meiosis in female mouse germ cells is somewhat reminiscent of the expression of different cyclin A paralogs during mitotic and meiotic G_2-M transitions in male mouse germ cells,[8] and the preferential use of CLB1 or CLB2 cyclin B paralogs in meiosis and mitosis in budding yeast.[9] Indeed, understanding how the transcriptional networks in mitotic and meiotic germ cells generate the complementary expression patterns of paralogous genes such as cyclin B1 and cyclin B3 may shed some light on how some of the fundamental differences between mitosis and meiosis are brought about and how developing germ cells change their mode of cell division from mitosis to meiosis.

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Spindle assembly checkpoint inactivation: A new role for phosphatases

Comment on: Visconti R, et al. Cell Cycle 2010; 9:564-9.

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In eukaryotic cells, successful cell division requires the separation and distribution of the proper number of chromosomes into each daughter cell during each mitosis. This process is accomplished by the bipolar mitotic spindle, which attaches microtubules to the kinetochores of paired mitotic chromosomes, facilitating their partition to each pole. Failures in the attachment and function of the mitotic spindle can lead to aneuploidy and genome instability, which contributes to the development of cancer. To ensure the fidelity of chromosome segregation, an elaborate Spindle Assembly Checkpoint (SAC) monitors the attachment of the mitotic spindle to the kinetochores and the generation of bipolar tension on each chromosome pair.[1] Unattached kinetochores or tensionless kinetochores activate the SAC, which blocks cell division by inhibiting the Anaphase Promoting Complex/Cyclosome (APC/C), a ubiquitin ligase required for mitosis. During each cell division cycle, the SAC inhibits the APC/C, and progression from metaphase into anaphase, until all the chromosomes are properly attached to the mitotic spindle, at which point the SAC is inactivated.[1]

The main effector of the SAC is the Mitotic Checkpoint Complex, composed of MAD2, BUB3 and BUBR1, which binds and inhibits CDC20, the substrate adapter for the APC/C that is required for the induction of anaphase via the degradation of Securin.[1] During each cell division cycle, the MCC binds CDC20, inhibiting the APC/C (and progression from metaphase into anaphase) until all the chromosomes are properly attached to the mitotic spindle, at which point the SAC is inactivated. It has been reported that SAC inactivation might occur through APC/C-mediated ubiquitination of CDC20, in a process independent of proteasomal degradation that leads to the dissociation of MAD2 and BUBR1 from CDC20.[2] However, more recent studies suggest that APC/C-mediated ubiquitination of CDC20 leads to CDC20 degradation and sustains the SAC in response to mitotic spindle failures, suggesting that other mechanisms must control SAC inactivation.[3,4]

In previous issue of *Cell Cycle*, Visconti et al. investigate other potential mechanisms controlling SAC inactivation and find evidence for the involvement of a protein phosphatase in licensing SAC resolution and entry into anaphase. Initially, their studies focused on the timing of SAC resolution, finding that the resolution of the SAC begins before the completion of the mitotic spindle and that the activation of the APC/C (in terms of Cyclin B degradation) lags behind the initial release of CDC20 from Mad2. In combination with previous studies, these results suggested that an additional factor—possibly controlling phosphorylation—is required for inactivation of the SAC, and, indeed, CDC20 phosphorylation changes dramatically during SAC inactivation.[5,6] Furthermore, the Mad2-CDC20 complex is stabilized in mitotic cells treated with proteasome inhibitors, but this complex can be dissociated by treatment with either cyclin-dependent kinase (CDK) or Aurora kinase inhibitors, which reduce CDC20 phosphorylation. Because degradation of CDC20 by the proteasome is not required for resolution of the SAC, these results suggest that the degradation of an additional factor is required to reverse the CDC20 phosphorylations that contribute to Mad2 binding.[3] Notably, Aurora A cannot phosphorylate CDC20, and proteolysis is unlikely to affect the activity of other kinases involved in the SAC.[7] Therefore, it appears that SAC inactivation requires the activity of a phosphatase, whose inhibitor is subject to proteasomal degradation.

The discovery of a role for a phosphatase and its inhibitor in SAC resolution is extremely important for our understanding of the cell division cycle, but the identity of these proteins remains a mystery. Previously, it was reported that the PP1 and PP2A phosphatases are activated by proteolysis to control mitotic exit;[8] however, the present study shows that the phosphatase activity required for SAC resolution is insensitive to concentrations of okadaic acid that inhibit both PP1 and PP2A. Additionally, no reports have addressed the potential identity of the phosphatase inhibitor that must be degraded to allow SAC inactivation by phosphatase activity. Nevertheless, Visconti et al highlight an emerging role for protein phosphatases in controlling the SAC and the metaphase to anaphase transition, and further work is required to identity the key factors in this regulatory pathway.

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New messages in the nuclear envelope

Comment on: Malhas A, et al. Cell Cycle 2010; 9:531-9.

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The past decade has witnessed a growing appreciation for the notion of the nuclear envelope (NE) as an organelle that integrates and transduces signals essential for metazoan development and tissue homeostasis, broadening the conception of the NE far beyond the traditional view of a fortress surrounding the nuclear contents.[1] Indeed, accumulating evidence that altered NE-based signaling causes of a diverse array of human diseases has attracted the attention of a growing number of biomedical researchers, ranging from basic cell biologists to physician-scientists.[1,2] One way that NE-localized proteins can regulate signaling is by controlling the availability of transcription factors, either by regulating their entry and exit through nuclear pores or through sequestration at the inner nuclear membrane. The mechanisms whereby NE proteins alter signaling are just beginning to be understood, however, so effects on transcription factors likely represent only the tip of the iceberg of a range of NE-localized modulatory mechanisms.

In the previous issue of *Cell Cycle*, Malhas, Saunders and Vaux provide evidence suggesting that the NE influences signaling via the regulation of miRNAs.[3] They performed miRNA microarray profiling in mouse embryonic fibroblasts lacking full-length lamin B1, a major component of the nuclear lamina of all or most mammalian somatic cells. This screen identified 20 miRNAs with significantly increased or decreased expression. Subsequent experiments, focused on the upregulated miRNA-31, identified candidate miRNA-31 targets, including genes involved in cell cycle progression, such as *Cdkn2a*. Reducing miRNA-31 levels using a chemically modified nucleic acid inhibitor in embryonic fibroblasts lacking lamin B1, which have greater proliferation rates than their wild type counterparts, decreased proliferation. Re-expression of lamin B1 in lamin B1 null fibroblasts rescued the miRNA-31 and proliferation phenotypes. The authors also identified a miRNA-31 binding site in the 3'UTR of p16Ink4a/p19Arf, potentially contributing to the cell cycle effects.

This intriguing study is the first piece of evidence implicating the nuclear envelope in the regulation of specific miRNAs. However, future work will need to address several critical questions. For example, the phenotype of *Lmnb1*$^{-/-}$ mice is not easily explained by a generalized defect in cell proliferation. These mice die at birth, with relatively selective defects of lung and bone, and the *Lmnb1*$^{-/-}$ fibroblasts used by Malhas et al. have severely misshapen nuclei as well as other defects.[4] miRNA profiling of several tissues during the development of *Lmnb1*$^{-/-}$ mouse embryos might clarify the cell types and developmental windows particularly sensitive to miRNA-31 expression levels and uncover whether altered expression of other miRNAs account for the relatively specific phenotypes of bone and lung. Furthermore, as *LMNB1* null humans have not been observed, the study of miRNAs in genetically modified mice or humans bearing a mutation associated with a human disease would provide more physiologically or "pathophsyiologically" relevant data.

In this regard, the results of Malhas et al. intersect with exciting work done by Ying-Hui Fu and colleagues. The Fu group has shown that a duplication of *LMNB1* with increased lamin B1 expression causes adult-onset autosomal dominant leukodystrophy, a disorder characterized by widespread myelin loss in the central nervous system.[5] They have further shown that miRNA-23 is a negative regulator of lamin B1 expression and that this miRNA contributes to myelin maintenance by downregulating lamin

B1.[6] Hence, lamin B1 may be at the center of a "signaling network" in which a miRNA regulates its own expression, which in turn regulates the expression of other miRNAs. The bidirectional interaction between lamin B1 and miRNAs adds to evidence implicating the nuclear envelope as a node that integrates and transduces a growing range of signals in development and disease.

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Hedgehog takes a new "RHOad" to angiogenesis

Comment on: Chinchilla P, et al. Cell Cycle 2010; 9:570-9.

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The Hedgehog proteins, highly conserved in vertebrates and invertebrates, are a family of secreted intercellular signaling molecules originally identified by genetic analysis of embryonic mutants of the *Drosophila melanogaster*.[1,2] Hedgehog ligands are critical in governing embryonic development and adult tissue homeostasis. They play an crucial role in the regulation in nearly all cellular processes including angiogenesis and neovascularization.[1-3] Hedgehog proteins signal via two, multi-transmembrane proteins, named PATCHED and SMOOTHENED.[4,5] In this receptor complex, PATCHED is the ligand-binding subunit, while SMOOTHENED is the signaling component. Upon binding of HH to its receptor PATCHED1, an inhibitory effect of PATCHED on SMOOTHENED is released, allowing SMOOTHENED to trigger a signaling cascade that activates the GLI transcription factors (GLI 1, 2 and 3), which are essential effectors for Hedgehog-mediated cellular effects.[5] In the absence of ligand, the Hedgehog signaling pathway is inactive. In this case, the transcription factors GLI undergo phosphorylation-dependent degradation. As a consequence, transcriptional activation of Hedgehog target genes is repressed. Pathway activation results in de-repression of SMOOTHENED, the central transducer for the signal, initiating a cascade that leads to the activation of GLI in the nucleus and regulation Hedgehog target genes.[5,6]

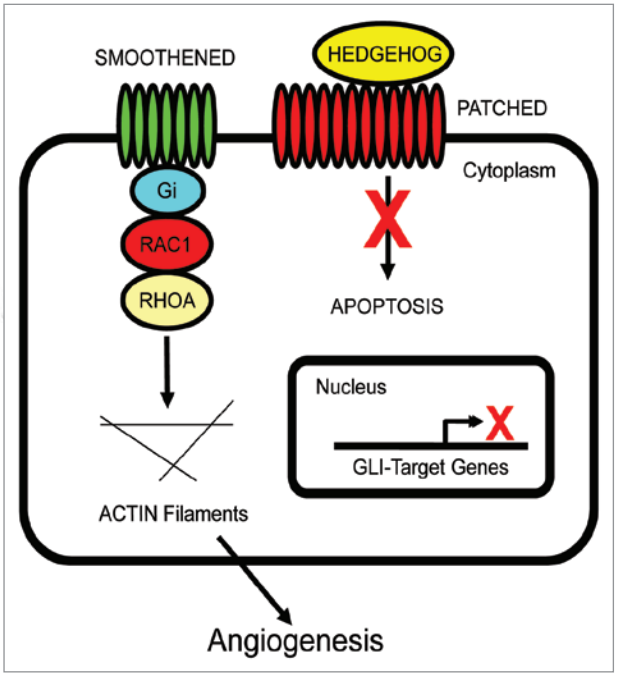

Figure 1. Hedgehog proangiogenic signaling pathway. Schematic representation of this novel proangiogenic Hedgehog signaling cascade in endothelial cells. Hedgehog promotes tube formation through a SMOOTHENED-Gi-RAC1-RHOA pathway. This cellular event is GLI independent and is accompanied by ligand dependent inhibition of PATCHED pro-apoptotic functions in endothelial cells.

In the study by Pilar Chinchilla and colleagues, the authors have identified a novel Hedgehog pathway independent of GLI transcription factors.[7] This pathway, activated by all three Hedgehog ligand (SONIC, INDIAN and DESERT), promotes vessel formation in endothelial cells. Angiogenesis is a coordinated multistep process involving endothelial cell proliferation, migration and formation tubular structures. The authors have found that endothelial cells do not respond to Hedgehog through the canonical pathway. None of the Hedgehog ligands were able to promote GLI transcriptional activity in endothelial cells. However, all three ligands increased endothelial cell tubulogenesis in a SMOOTHENED-dependent manner. Hedgehog induces the typical elongated morphology of activated endothelial cells. Analysis of the mechanism showed that Hedgehog requires an intact Gi protein function to form tubes. This Hedgehog stimulated SMOOTHENED-Gi axis activates through RAC1 the small GTPase RHOA and the formation of actin stress fibers in endothelial cells, a step that is key for tube organization. In addition to regulating the actin cytoskeleton, the Hedgehog ligands contribute to the angiogenic process by increasing endothelial cell survival through the inhibition of the pro-apoptotic effect of unligated PATCHED receptor. Interestingly, this effect is a SMOOTHENED-independent manner and

involves the reduced CASPASE activation in response to pro-apoptotic stimuli (e.g., serum starvation). Thus, this study defines a novel non-canonical Hedgehog pathway and shifts the paradigm of Hedgehog signaling by supporting the existence of GLI-independent regulated cellular functions. Together these findings not only provide a novel insight into the biology of the Hedgehog cascade but could also serve as foundation for the development novel therapeutics and diagnostic approaches for diseases dependent on an active Hedgehog pathway.

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A direct, non-canonical pathway for Hedgehog proteins in the endothelium

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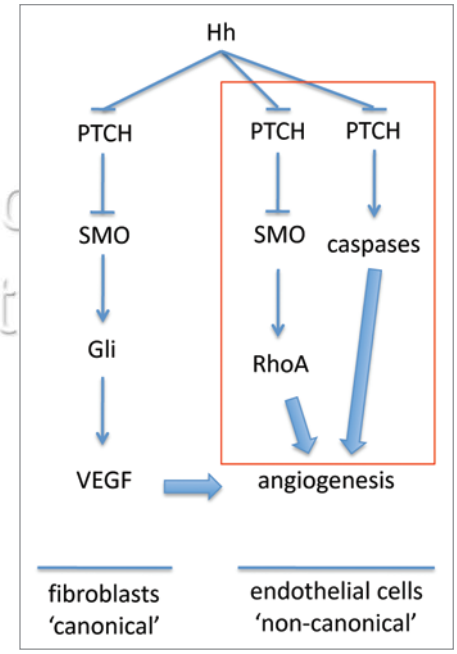
The hedgehog (Hh) signaling pathway is implicated in a broad spectrum of activities, ranging from embryonic patterning to promoting angiogenesis in the adult.[1,2] A common canonical paradigm has largely prevailed to explain the mode of action of Hh in such diverse types of processes. Hh ligands (Shh, Ihh and Dhh) bind to and inhibit the inhibitory action of the Hh receptor (Patched-1 or PTCH1) on Smoothened (SMO), the central transducer of the Hh pathway. The rank order ofpotency is Shh >> Ihh > Dhh.[3] In the classical or canonical pathway, all signals carried by Hh proteins converge to a common family of transcription factors, termed Gli. Inhibition of phosphorylation-dependent degradation of Gli-2 and -3 proteins by Hh leads to the widespread activation of the transcription of not only cell cycle, survival and pro-angiogenic factors, but also of various members of the Hh pathway cascade, like ptch1 and Gli1.[3,4] The latter activation represents the hallmark of the classical response to Hh stimulation.

In the classical angiogenic paradigm, the inductive effect of Hh proteins on the formation of the new vasculature relies on the canonical action of Hh proteins on stromal fibroblasts. In the stroma, the Hh activity results in a robust Gli-dependent stimulation of VEGF and other key angiogenic factors, whose action impacts the adjacent endothelium to promote angiogenesis.[5] Although there is emerging evidence for the existence of non-canonical modes of action,[6] no data showing a direct effect of Hh on the endothelium has been documented as of yet.

In the previous issue of *Cell Cycle*, Chinchilla et al. elegantly show that a direct action of Hh proteins on endothelial cells (ECs) leads to organization of actin stress fibers and orchestrates tubulogenesis through a non-canonical, Gli independent mechanism. Chinchilla et al. studied the effect of Hh proteins on human umbilical cord endothelial cells (HUVECs) or human cardiac microvascular endothelial cells (HMVECs). Unexpectedly, these ECs were responsive to Hh proteins, as they promoted tubulogenesis in 3D cultures. However, *ptch1* or *Gli1* were not transcribed, suggesting that the canonical pathway was not active. In contrast to stimulatory strength differences observed for Hh proteins (Shh>>Ihh>Dhh) through the canonical mode of action, all Hh proteins promoted tubulogenesis with similar strength in the ECs. To dissect out the mechanism of action of Hh proteins on ECs, Chinchilla et al. employed chemical compounds, toxins, dominant negative proteins and siRNA to inhibit/neutralize specific points in the Hh pathway. Their findings indicate that the direct, non-canonical induction of tubulogenesis appeared to be dependent on the activation of SMO and heterotrimeric G proteins. Activation of SMO led to the activation of the small GTPase RhoA, which induced a pronounced reorganization of the actin cytoskeleton into stress fibers. This is the first time Hh isoforms have been shown to stimulate GTPase RhoA in endothelial cells via SMO and heterotrimeric Gi proteins. Secondly, Chinchilla et al. went on to show that Hh proteins are also implicated in another novel, direct, non-canonical effect, this time independent of the action of SMO. This mechanism involves the inactivation of the apoptotic role of PTCH1 mediated by caspase activation.



A novel Gli-independent, direct, non-canonical paradigm is depicted (red box).

Taken all together, the work by Chinchilla et al. describes novel, direct, Gli-independent (SMO-dependent and SMO-independent) roles for Hh proteins on the endothelium. This work also sheds light on a potential link between RhoA and the Hh pathway. It would be intriguing to test if the RhoA-Hh link specifically takes place in the endothelium, or represents a more generalized process. It would also be interesting to determine how significant the contribution of this novel non-canonical pathway is in an in

vivo setting, where canonical, paracrine, pro-angiogenic effects also take place. Future work focused on the interaction and coordination of these two pathways may lead to new insights regarding angiogenic processes. This study further opens the possibility for the exploration of novel anti-angiogenic targets, whose inhibition may impinge on the action of Hh on the endothelium directly. Overall, Chinchilla et al. offers a comprehensive study highlighting the biological relevance of non-canonical Hh signaling in endothelial cells during angiogenic events.

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p63 and canonical Wnt signaling

Comment on: Drewelus I, et al. Cell Cycle 2010; 9:580-7.

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p63 as master regulator of epithelial stemness

ΔNp63α has been described as a "master gene" for maintenance of epithelial stemness in all stratified epithelia, at least in part by blocking apoptosis and differentiation. Thus, the human developmental Ectrodactyly-Ectodermal Dysplasia syndromes, marked by various mutations in the p63 gene, as well as the phenotype of the global p63-null mice, put p63 in the center of regulating organogenesis of skin and skin adnexae as well as breast, prostate and urothelium. Moreover, ΔNp63α has also a role in an earlier phase of development by promoting ectoderm formation and suppressing mesoderm induction, as shown e.g. in mouse ES cells and in *Xenopus laevis*.

ΔNp63α in tumor progression

An aberrant overexpression of ΔNp63 via gene amplification or increased protein translation is present in many epithelial carcinomas, such as squamous cell carcinoma and carcinomas of the bladder, breast and prostate. However, during tumor progression clinical studies repeatedly showed subsequent loss of ΔNp63α at least in some tumor types, which correlated with increased invasion and metastases and poorer prognosis for the patient. For example, studies in bladder carcinoma showed that 50% of the cases exhibited aberrantly high levels of ΔNp63α protein. However, these were only non-invasive superficial carcinomas, while invasive tumors had lost p63 expression.[1,2,3] Similar results were seen for carcinomas of the endometrium and esophagus. These results were paralleled in vitro in squamous cell carcinoma lines where p63 knockdown caused decreased cell adhesion and increased cell migration and was associated with the induction of an invasive transcriptome.[4] Thus, the effects of ΔNp63α in tumor formation appear stage dependent.

The canonical Wnt signaling pathway in development

The three major Wnt signaling pathways are involved in nearly every aspect of embryonic development and in adult tissue homeostasis. The canonical Wnt pathway governs specification of cell identity, embryonal axis determination and regulation of cell proliferation and differentiation. It centers around beta-catenin, that upon stabilization binds to TCF/LEF transcription factors and leads to the activation of multiple Wnt target genes involved in development. In the default/inactive state, β-catenin is continuously degraded in the cytoplasm, and TCFs recruit corepressors (i.e., Groucho) to Wnt target genes. Conversely, upon Wnt ligand binding, β-catenin escapes its degradation fate, accumulates in the nucleus and replaces the TCF-bound co-repressors to occupy Wnt target genes. Once bound, β-catenin functions as a scaffold to recruit auxiliary machinery of co-activators that are involved in chromatin remodeling to induce Wnt target gene expression.

Specifically, the development of apical ectodermal ridge (AER), essential for limb development in mammals, depends on the activity of the canonical Wnt pathway in the underlying mesenchyme.[5]

The canonical Wnt signaling pathway in cancer

A dysregulation of the canonical Wnt signaling in cancer is well established. Constitutively active Wnt signaling due to inappropriate stabilization of β-catenin is the central theme here. This is achieved by loss-of-function mutations of components of the cytoplasmic destruction complex, such as the APC gene in the Familial Adenomatous Polyposis (FAP) syndrome leading to colon cancer, mutations in Axin1 or Axin2 in hepatocellular adenocarcinoma,or non-degradable, mutant β-catenin in tumors of colon, hair follicles and breast. Moreover, aside from tumor initiation, β-catenin signaling is also very important during tumor progression since active Wnt signaling is heavily involved in cell migration and invasion and can cause epithelial-to-mesenchymal transition (EMT).[6,7]

Partial overlap in expression and activity between p63 and components of canonic Wnt signaling

During development, specific tissues comprised of skin, hair follicles, mammary glands and limb buds (AER) exhibit overlap in expression and function between ΔNp63α and components of Wnt signaling, suggesting a mechanistic interaction. Some overlap can also be seen in tumorigenesis and tumor invasion, as mentioned above. However, the paper by Drewelus et al. in the previous issue of *Cell Cycle* is only the second published report that directly addresses a putative crosstalk between ΔNp63α and the

β-catenin-Wnt signaling pathway. The first such connection came from a transient overexpression study by Patturajan et al. in 2002[8] reporting a positive synergism between the two. These authors concluded that ΔNp63 induces Wnt signaling by inhibiting GSK3β (another component of the cytoplasmic β-catenin destruction complex) via binding to the subunit B56α of PP2A, thereby promoting nuclear accumulation of β-catenin. However, studies of human tumors did not find a correlation between overexpression of ΔNp63 and nuclear accumulation of β-catenin. In the second study reported here, Drewelus et al., using transient overexpression of ΔNp63α initially confirmed this cooperativity in the same cell system in vitro (HEK293). Moreover, inducible LEF-1 also cooperated with overexpressed ΔNp63α in vivo, generating secondary axis formation due to ectopic hyperactivation of Wnt signaling in *Xenopus laevis* embryos. Surprisingly though, downregulation of endogenous levels of ΔNp63α in these authors' hands equally activated Wnt signaling, as evidenced by a Wnt reporter gene and endogenous Wnt target genes, mainly Axin2. In addition, ΔNp63α was capable of physical interaction with multiple TCF/LEF family members upon ectopic expression. The contact was mapped to the central DNA-binding domain of ΔNp63α and the HMG domain of LEF-1. Of note, neither levels nor phosphorylation status of β-catenin changed when Drewelus et al knocked down ΔNp63α, arguing for an additional interactive mechanism of Wnt signaling activation taking place at the transcriptional level. While a limited study, what makes it interesting is that the authors put forward an elegant simple model to plausibly explain the seeming paradox, i.e., how ΔNp63α overexpression and downregulation could both elicit the same net effect on Wnt signaling. By being a physical connector between TCF/LEF and putative co-repressors of transcription, ΔNp63α—when overexpressed—would squelch the limiting concentrations of the co-repressors in a separate complex, thereby artifactually derepressing TCF/LEF target genes. This would mimic the p63 knockdown situation. Similar explanations have been invoked previously for other transcriptional regulators. For example, for Gal4 in yeast and Fos/Jun or more recently E2F1 in mammalian cells, where ambiguous results were obtained after comparing overexpression with dominant negative blockade or knockout. As such, both overexpression of full length E2F1 or E2F4 and their dominant-negative mutants resulted in displacement of the active Rb/E2F complexes from chromosomal sites and subsequent target gene derepression imposed by associated corepressors, e.g. HDAC or BRG1/BRM.[9,10] Although it remains to be investigated, the model put forward that ΔNp63α antagonizes Wnt-induced transcription opens up the possibility that the limb bud phenotype due to AER regression that is seen in p63KO mice is due to hyperactivation of Wnt signaling. This scenario would be consistent with the limb truncations seen with stabilized β-catenin (Hill 2006). A general repressor role of ΔNp63 on the Wnt pathway, at first glance, seems difficult to reconcile with the observation of similar skin appendage abnormalities in both p63KO and LEF1 KO 11 or Tcf3/4 KO mice.[12] However, it was suggested that the role of Tcf3/4 in the epidermis consists at least partially in a repressor function, and this would be in line with an auxiliary role of ΔNp63 in TCF-mediated repression.[12] Moreover, the antagonistic model is attractive for tumor progression since it could explain the association between loss of ΔNp63 expression and epithelial mesenchymal transition (EMT) of invading carcinomas, a phenomenon in part dependent on Wnt signaling.

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New insights into KLF8-mediated transactivation

Comment on: Urvalek AM, et al. Cell Cycle 2010; 9:601-11.

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The Krüppel-like factor (KLF) family of transcription factors control diverse processes, including regulation of the cell cycle, differentiation, apoptosis, and tumorigenesis. KLF8 is no exception, and previous work has demonstrated a role for KLF8 in proliferation,[1] PI3K/Akt signaling,[2] oncogenic transformation[3] and cell invasion.[4] As would be expected for a transcription factor with such a wide variety of functions, expression and activity of KLF8 is highly regulated, and in a manner that is only recently coming into focus. For example, activation of Focal Adhesion Kinase (FAK), a key effector of integrin signaling, results in increased expression of KLF8, at least partially through activation of the PI3K/Akt signaling pathway and increased expression of Sp1.[1,4] Post-translational modification of KLF8 via SUMOlyation at lysine 67 attenuates the ability of KLF8 to both activate the Cyclin D1 gene and repress the Klf4 gene.[5] In addition, closely-related family members KLF1 and KLF3 activate and repress expression of KLF8, respectively.[6]

Less is known about how KLF8 modulates expression of its target genes. KLF8 has been shown to interact with the co-repressor CtBP, and mutation of the N-terminal sequence PVDLS to PVASLS markedly decreases the strength of this interaction in vitro.[7] Similarly, KLF4 interacts with CtBP[8] and can both activate and repress target genes, depending on the cellular context and cofactors involved (see **Fig. 1**).[9] KLF4 recruits p300/CBP in order to activate expression of the IAP gene and promote acetylation of nearby histones.[10] Thus, it is possible that KLF8 may act via a similar mechanism to transactivate the Cyclin D1 promoter.

Indeed, in a recent issue of *Cell Cycle* (Volume 9, Issue 3), work from Urvalek, Wang, Lu and Zhao reveal that KLF8 interacts with the co-activators p300, CBP, and P/CAF.[11] Moreover, through detailed mutational analysis, the authors identify Q118 and Q248 as key residues in mediating this interaction. The double point mutant KLF8-Q118N/Q248N is unable to interact with p300, CBP or P/CAF in an immunoprecipitation assay. Moreover, KLF8-Q118N/Q248N is significantly less able

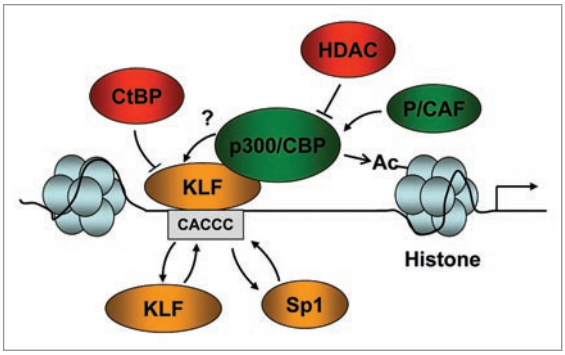

Figure 1. A general model for transcription mediated by members of the KLF family. Krüppel-like factors bind to their consensus CACCC motif on target genes and recruit the co-activators p300 or CBP. These co-activators acetylate nearby histones and activate transcription. The function of this complex can be modulated by histone deacetylases (HDACs) or the p300/CBP associated factor (P/CAF). Depending on the gene context, KLFs can also recruit co-repressors, such as CtBP, and repress transcription. In addition, KLF family members often compete with each other or the closely related protein Sp1 for binding these CACCC consensus sequences, adding yet another level of regulation of KLF transcriptional activity. The role of p300 and CBP on KLFs acetylation and the functions of KLFs acetylation need to be further examined.

to activate a Cyclin D1-Luciferase reporter construct, increase expression of the Cyclin D1 protein or promote proliferation.

p300, CBP and P/CAF all contain a HAT domain which catalyzes acetylation of nearby histones as well as other proteins. Consistent with this role, the authors demonstrate that overexpression of wild-type KLF8 results in increased histone acetylation on the Cyclin D1 promoter, whereas KLF8-Q118N/Q248N is markedly less able to increase acetylation. In $p300^{-/-}$, $CBP^{-/-}$ and $P/CAF^{-/-}$ cells, KLF8 is significantly less able to activate a Cyclin D1 reporter construct. Restoration of p300 or P/CAF expression at least partially restores activation of this reporter. Interestingly, CBP has the opposite effect, and actually further inhibits the ability of KLF8 to activate the Cyclin D1 reporter. In fact, even in wild-type MEF cells, overexpression of CBP blocks the ability of KLF8 to activate the reporter. Although it is unclear whether the effects of these coactivators on the Cyclin D1 reporter are exerted exclusively through KLF8 or through neighboring transcription factors, these data clearly suggest differential roles for p300 and CBP in regulation of the Cyclin D1 gene.

These new data are exciting for several reasons. First, this is the first publication to demonstrate co-activators used by KLF8 for modulating transcription and promoting histone acetylation. KLF1, KLF4, KLF5, KLF6 and KLF13 have all been shown to interact with p300, CBP, or P/CAF, suggesting that recruitment of these cofactors is part of a general mechanism for the function of transcription factors in the Krüppel-like factor family. Given that p300/CBP and P/CAF often acetylate the transcription factors they interact with, it will be interesting to test whether KLF8 is acetylated and what its function might be. Second, the authors identify two key residues in the KLF8 putative transactivation domain that are critical for interactions with these cofactors. The double point mutant Q118N/Q248N will certainly be useful for further studies in teasing out the precise molecular mechanisms of KLF8-mediated transcription. Finally, the authors demonstrate differential roles for p300 and CBP in modulating Cyclin D1 transcription, although the reason for this result is not entirely clear. It is worth noting that KLFs have distinct roles in cell proliferation and human cancers. KLF4 is a tumor suppressor in the GI cancers and repress CyclinD1 expression, while KLF8 promotes transformation and activates CyclinD1 genes, suggesting that the roles of p300/CBP are highly context dependent. It will be interesting to investigate whether CtBP plays any role in KLF8-mediated regulation of the Cyclin D1 gene, and if p300 or CBP affects recruitment of CtBP or vice-versa. This new article from Urvalek and colleagues is quite interesting and is likely only the beginning of many subsequent publications on the molecular mechanisms of KLF8-mediated transcription.

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KLF8 sets the pace for the cell cycle through interactions with p300 and PCAF

Comment on: Urvalek AM, et al. Cell Cycle 2010; 9:601-11.

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Krüppel-like factors (or KLFs) are a family of at least 17 transcription factors which share homology in their three C2-H2 zinc finger DNA binding domains. KLF8 has a well established role in regulating cell cycle progression and oncogenic transformation.[1,2] KLF8 can negatively regulate the globin[3,4] and E-cadherin genes,[5] and positively regulate genes such as cyclin D1 1. The mechanism for KLF8 negative regulation has been studied, and requires contact with the C-terminal Binding Protein (CtBP) corepressor.[3] The means by which KLF8 positively regulates genes was previously unknown. In the previous issue of *Cell Cycle*, Urvalek et al. demonstrate that KLF8 promotes histone acetylation at the cyclin D1 promoter, and that transactivation of the promoter by KLF8 requires recruitment of the p300 or p300/CBP associated factor (PCAF) co-activators of the histone acetylase family.[6] The Q118 and Q248 amino acid residues are required for KLF8 to

transactivate the cyclin D1 promoter and for physical interactions between KLF8 and the co-activators.

KLF8 activates the cyclin D1 gene, promoting cell cycle progression through the G_1 phase.[1] Other KLF factors also have demonstrated roles in controlling the cell cycle. For example, the cyclin D1 gene is a direct transcriptional target of KLF13 in the heart.[7] EKLF (erythroid Krüppel-like factor or KLF1) was the first KLF family member to be described.[8] Cells lacking EKLF/KLF1 have a defect in S-phase entry, likely due to reduced expression of E2f2 and E2f4, which are directly activated by EKLF.[9,10]

The fact that KLF8 recruits p300 and PCAF increases the evidence for and number of KLF factors potentially using a common mechanism of transactivation. EKLF/KLF1, KLF2, KLF4, KLF5, KLF6, KLF8, KLF11 and KLF13 are now known to recruit co-activators of the histone acetylase family (see references in Urvalek et al. and ref. 11). It is not always clear in these examples whether acetylation of the KLF transcription factor, or acetylation of histones as is seen here for KLF8, is the primary mechanism for gene activation. KLF8 may be unique in that its transactivation domain directly interacts with p300/PCAF, although the KLF domain required for such interactions is not well defined in all cases. It will be interesting to investigate in the future whether direct connections, such as those documented by Urvalek et al. for KLF8,[6] exist between other KLFs, members of the histone acetylase family, and cell cycle progression.

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